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Label-free proteomics reveals serum proteins whose levels differ between pancreatic ductal adenocarcinoma patients with short or long survival

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Abstract

Pancreatic ductal adenocarcinoma is the most common and aggressive type of pancreatic cancer, with a 5-year survival rate that is less than 10%. New biomarkers to aid in predicting the prognosis of pancreatic ductal adenocarcinoma patients are needed. Previous proteomic studies have to a great extent focused on finding proteins of value for the diagnosis of pancreatic ductal adenocarcinoma. There is a lack of studies that have profiled the serum or plasma proteome in order to discover candidates for new prognostic biomarkers. In this study, we have used ultra-performance liquid chromatography–ultra-definition mass spectrometry to analyze the serum samples of 21 pancreatic ductal adenocarcinoma patients with short or long survival. Statistical analysis discovered 31 proteins whose expression differed significantly between pancreatic ductal adenocarcinoma patients with short or long survival. Pathway analysis discovered multiple canonical pathways enriched in this data set, with several pathways having roles in inflammation and lipid metabolism. The serum proteins identified here, which include complement components and several enzymes, could be of value as candidates for new noninvasive prognostic markers.

Keywords

Pancreatic ductal adenocarcinoma, serum, proteomics, prognosis, proteins

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Introduction

Pancreatic cancer comprises several distinct neoplasms arising from the pancreas and is the seventh most common cause of cancer death worldwide. Pancreatic ductal adenocarcinoma (PDAC) accounts for around 85% of all pancreatic cancer cases and is the most aggressive type of pancreatic cancer, with a very poor prognosis and most patients presenting with metastatic disease. Each year, around 330,000 new cases of PDAC are diagnosed worldwide, with an almost equal number of deaths.¹ The 5-year survival rate for pancreatic cancer patients is under 10%. The only possible way to cure PDAC is through surgery, although less than 20% of patients present with potentially resectable tumors. For these patients, the 5-year survival is only increased to

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20%, which leads to treatment for most patients being focused on palliative care.^{2,3}

Prognosis remains poor even for patients who undergo radical surgery, with patients being faced with significant morbidity, and up to 80% of patients develop recurrence.^{4,5} PDAC presents with nonspecific symptoms, such as weight loss and abdominal pain, making it difficult to diagnose. Patients are often elderly and in poor overall health, and PDAC is also associated with multiple comorbidities, which further complicate treatment.⁶ New biomarkers to aid in the prediction of prognosis, treatment response, and follow-up of PDAC are needed, as carbohydrate antigen 19-9 (CA 19-9) is the only routinely used serum-based biomarker for PDAC.⁷ CA19-9 is an independent prognostic variable and high levels of CA19-9 are correlated with decreased survival in PDAC patients. However, as CA19-9 levels can also be elevated due to other benign and malignant conditions, caution must be used when interpreting results.^{8,9}

Mass spectrometry is often used to analyze the proteome for potential biomarker candidates. Proteins detectable in serum can be measured from easily obtained blood samples and are therefore ideal as biomarkers.¹⁰ A peptide panel that would be cost-effective, able to detect early disease, and able to be run even when symptoms are general and non-specific would be ideal. Mass spectrometry-based proteomic analysis of serum gives information about the expression levels of hundreds of proteins simultaneously in a single experiment, and the results can be further analyzed to obtain candidates for diagnostic and prognostic use. Furthermore, information about dysregulated pathways in the disease can also be obtained, and this information can be used to design studies to gain mechanistic insights into disease progression and therapeutic target discovery.

Previous studies aiming to identify biomarkers for PDAC patients have to a large extent focused on finding proteins of use in the diagnosis of PDAC and have investigated the differences in protein expression between serum from PDAC patients and healthy controls or between PDAC tissue and healthy pancreatic tissue.^{11–17} Several studies have investigated if specific proteins are linked with prognosis and survival in PDAC patients, although these have mainly utilized tissue samples. A study by Winter et al. hypothesized that tumor biology was the main driver of survival groups separated by a minimum of 1.5 years between short- and long-term survival. The authors used tissue microarrays comprised of tumor samples associated with short- and long-term survival after PDAC resection. They subsequently discovered that the proteins, mesothelin (MSLN) and mucin-1 (MUC1) (which has previously been identified as a protein carrier of the CA 19-9 antigen) were predictors of early cancer-

specific mortality.^{18,19} Few studies have analyzed serum samples from PDAC patients in order to find proteins linked to survival and prognosis. One study analyzed levels of Dickkopf-1 (DKK1) in the serum of PDAC patients by enzyme-linked immunosorbent assay (ELISA) and found that DKK1 levels increased with increasing tumor stage. DKK1 expression in tissues correlated with its expression in serum and patient survival was lower in patients with higher DKK1 levels.²⁰ Another study using 2D electrophoresis found that serum levels of cofilin-1 increased with PDAC progression, indicating that cofilin-1 could be a potential prognostic marker of PDAC.²¹

Prognostic markers for PDAC could help to predict patients' prognosis and subsequently guide treatment decisions. By identifying those patients with a poor prognosis, patients could be selected for more aggressive treatment. Biomarkers associated with poor prognosis also offer the potential to become targets for new therapies.²² In this pilot study, we have analyzed the serum proteome of a small cohort of PDAC patients by mass spectrometry and compared the protein expression between patients with short- and long-term survival, in order to discover proteins that could be of value as new candidates for prognostic biomarkers. We discovered multiple proteins whose expression differed significantly between the groups, which after further studies and validation could be of clinical utility.

Materials and methods

Patient samples

This study included preoperative serum samples from 22 patients with PDAC who underwent pancreaticoduodenal resection at the Department of Surgery, Helsinki University Hospital, between 2001 and 2011. Bilirubin levels were measured preoperatively and all jaundiced patients underwent an endoscopic procedure and received a biliary stent prior to operation. At the time when the serum samples for this study were collected, the bilirubin levels of all patients had normalized (data not shown). Of the patients in this study, 12 patients were male and 10 were female, and their age ranged from 54 to 79. The patients were chosen according to their postoperative survival, with 11 patients dying within 1 year after surgery (short-term survival) and 11 patients surviving at least 5 years after surgery (long-term survival). Detailed patient characteristics including tumor stage, grade, location, and patient age, adjuvant therapy status, survival, and cause of death are given in Supplementary Table 1. Serum samples were stored at -80°C until further processed as described below. This study was approved by the Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/06, extension TMK02 §66

17.4.2013) and was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients included in this study.

Sample processing and digestion

The serum samples were processed as previously described.^{23,24} Briefly, the samples were thawed before top 12 protein depletion was performed using the TOP12 protein depletion kit (Pierce, ThermoFisher, MA, USA) according to the manufacturer's instructions. The total protein concentration was determined in the depleted serum using the Pierce BCA assay kit (Pierce, ThermoFisher, MA, USA). The amount of serum equivalent to 100 µg protein was aliquoted and dried, after which it was dissolved in Tris buffer containing urea. Dithiothreitol (DTT) was added to the samples, which were shaken for 1 h, after which iodoacetamide was added. Samples were shaken another hour at room temperature, after which additional DTT was added and the samples were shaken again and then diluted using mQ. Trypsin was added at a ratio of 1:50 trypsin to protein. Digestion was carried out at 37°C overnight, and the next day 30 µg of tryptic peptides were cleaned and dissolved in 0.1% formic acid with 12.5 fmol/µL of Hi3 spike-in standard peptides (Waters, MA, USA).

Ultra-performance liquid chromatography–ultra-definition mass spectrometry and quantification

UPLC–UDMS^E. Ultra-performance liquid chromatography–ultra-definition mass spectrometry (UPLC–UDMS^E) was performed as previously described.^{23,24} In summary, 4 µL of each sample (around 1.4 µg protein) was injected into a nanoACQUITY UPLC system (Waters Corporation, MA, USA). TRIZAIC nanoTile 85 µm × 100 mm HSS-T3u wTRAP was used for separation. Data were acquired in data-independent acquisition fashion using UDMSE mode with a Synapt G2S HDMS (Waters Corporation, MA, USA). Data were collected in the range of 100–2000 m/z, scan time of 1 s, ion-mobility spectrometry (IMS) wave velocity of 650 m/s, and collision energy was ramped from 20 to 60 V. Calibration was performed using Glu1-Fibrinopeptide B MS2 fragments. Glu1-Fibrinopeptide B precursor ions were used as a lock mass during the runs. All samples were run in triplicates.

Data analysis. Data analysis and label-free quantification were performed as previously described.^{23–26} Progenesis QI for proteomics automatically performs match-between-runs (aligning results from different runs), which results in increased reliability and reproducibility. The parsimony principle was used to group the

proteins, and peptides unique to the protein were also reported, meaning that the protein hits were reported as the minimum set that comprises all observed peptides. Due to over-stringency, Progenesis QI for proteomics does not follow a strict parsimonious approach.²⁷ If two proteins are found with common peptides, the protein with fewer peptides is subsumed into the protein with more peptides. Relevant proteins are listed as a group under the lead protein, which is the one with the highest coverage or score. Quantitation is performed with the lead identity peptide data. For more details, see Nonlinear Dynamics' website (www.nonlinear.com). The mass spectrometry proteomics data generated in this project have been deposited to the Proteome Xchange Consortium via the PRIDE^{28,29} partner repository with the data set identifier PXD005144.

Further analysis. The differences between the two groups (short and long survival) were analyzed using the Mann–Whitney U test. Principal component analysis (PCA) was performed using Progenesis QI for proteomics. Data were normalized by Pareto scaling and hierarchical clustering was performed using the program MetaboAnalyst, version 4.0.^{30,31} For generating the heatmap, the following parameters were used: distance measure: Pearson, clustering algorithm: Ward, and “autoscale features.” Pathway analysis was performed using ingenuity pathway analysis (IPA) (QIAGEN Bioinformatics, Redwood City, CA, USA). Only those proteins with a *p*-value of <0.05 were used for pathway analysis. Receiver operating characteristic (ROC) curves were obtained using SPSS version 25.0 (IBM SPSS Statistics, IBM Corporation, Armonk, NY), and the results are reported as area under the ROC curve (AUROC) values.

Results

Protein identification

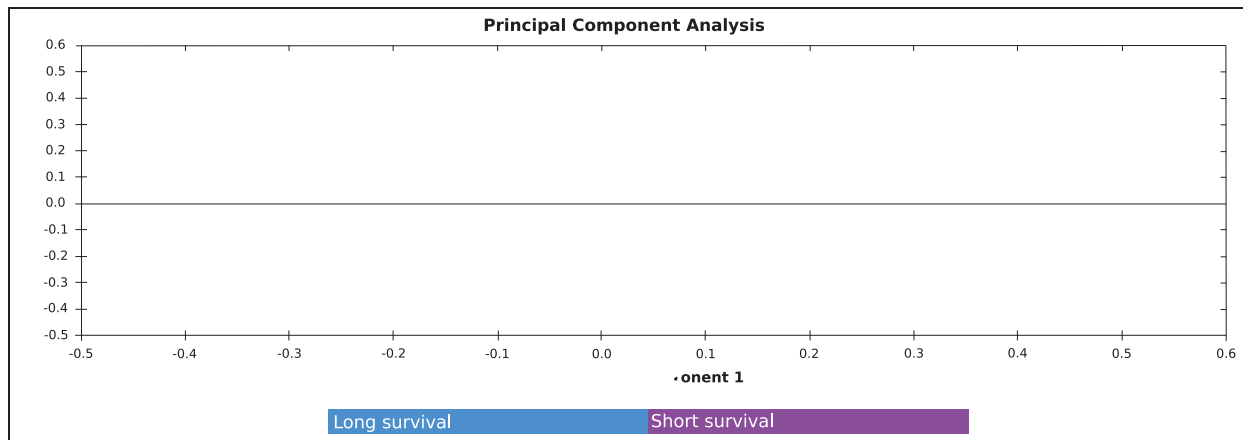
In this study, we analyzed serum samples from a total of 21 PDAC patients (after the exclusion of one sample that did not normalize, marked in Supplementary Table 1). The samples were divided into groups depending on patient survival, short (*n* = 10) or long (*n* = 11). A total of 140 proteins with two or more unique peptides were quantified. The full list of proteins with relevant data is given in Supplementary Table 2. Proteins with *p*-values greater than 0.05 were not considered to be significantly different between the two groups.

Differentially expressed proteins

A total of 31 proteins passed the requirement of having a *p*-value of less than 0.05 when analyzed by the Mann–Whitney U test (Table 1) and their expression was

Table 1. The 31 serum proteins that passed the cutoff of a Mann–Whitney *p*-value of less than 0.05 when samples from PDAC patients with short or long survival were compared.

Accession	Peptide count	Unique peptides	Confidence score	Mann Whitney <i>p</i> -value	Fold change (short/long survival)	AUROC	AUROC 95% CI	Protein name	Gene name
O43866	5	5	30.9	1.59E-02	-10.6	0.782	0.585–0.978	CD5 antigen-like	CD5L
P35908	3	2	18.6	1.58E-02	5.7	0.782	0.579–0.985	Keratin_type II cytoskeletal 2 epidermal	KRT2
P53602	2	2	11.3	2.64E-02	-3.8	0.755	0.543–0.966	Diphosphomevalonate decarboxylase	MVD
Q2741	6	3	44.7	2.24E-02	3.7	0.764	0.550–0.977	C-reactive protein	CRP
Q9BXR6	3	2	17.8	1.33E-02	-3.0	0.791	0.583–0.999	Complement factor H-related protein 5	CFHR5
P80108	12	9	74.1	1.59E-02	-2.9	0.782	0.565–0.999	Phosphatidylinositol-glycan-specific phospholipase D	GPLD1
P35527	18	15	163.6	5.08E-03	2.3	0.836	0.660–1.000	Keratin_type I cytoskeletal 9	KRT9
P09871	38	34	324.2	3.10E-02	2.0	0.745	0.512–0.979	Complement C1s subcomponent	C1S
H0Y1W9	15	3	107.7	9.16E-03	1.9	0.809	0.617–1.000	Uncharacterized protein (Fragment)	
P2042	10	4	74.0	2.64E-02	-1.9	0.755	0.517–0.993	Hemoglobin subunit delta	HBD
P50508	3	2	17.4	3.63E-02	-1.9	0.736	0.511–0.962	Phospholipid transfer protein	PLTP
P02654	10	9	64.7	1.89E-02	-1.8	0.773	0.556–0.990	Apolipoprotein C-I	APOC1
P04264	30	22	235.5	1.11E-02	1.7	0.800	0.596–1.000	Keratin_type II cytoskeletal I	KRT1
P0C0L5	167	7	2034.1	4.22E-02	1.6	0.727	0.506–0.948	Complement C4-B	C4B
P02748	51	42	453.5	1.33E-02	1.6	0.791	0.574–1.000	Complement component C9	C9
P27169	24	20	286.1	4.90E-02	-1.6	0.718	0.493–0.944	Serum paraoxonase/arylesterase I	PONI
P02750	37	31	321.7	1.59E-02	1.5	0.782	0.585–0.979	Leucine-rich alpha-2-glycoprotein	LRG1
P13796	6	3	38.3	4.90E-02	1.5	0.718	0.494–0.942	Plastin-2	LCPI
P05452	25	21	203.0	1.89E-02	-1.5	0.773	0.570–0.976	Tetranectin	CLEC3B
P05090	16	13	148.4	2.64E-02	-1.4	0.755	0.542–0.967	Apolipoprotein D	APOD
P51884	20	12	193.1	2.24E-02	1.4	0.764	0.534–0.993	Lumican	LUM
Q9NZP8	4	2	32.4	4.22E-02	1.4	0.727	0.492–0.962	Complement C1r subcomponent-like protein	C1RL
P07357	37	33	278.6	4.22E-02	1.3	0.727	0.506–0.948	Complement component C8 alpha chain	C8A
P07360	14	14	148.6	1.89E-02	1.3	0.773	0.542–1.000	Complement component C8 gamma chain	C8G
A0A075B6Z2	4	3	15.4	4.22E-02	-1.3	0.727	0.492–0.963	T cell receptor alpha joining 56 (Fragment)	TRAJ56
P14902	3	3	16.4	3.10E-02	1.2	0.745	0.520–0.971	Indoleamine 2,3-dioxygenase 1	IDO1
P07358	47	40	432.5	4.22E-02	1.2	0.727	0.479–0.976	Complement component C8 beta chain	C8B
P01024	536	478	3705.9	2.64E-02	1.2	0.755	0.521–0.988	Complement C3	C3
P02749	57	52	389.6	4.90E-02	1.2	0.718	0.470–0.966	Beta-2-glycoprotein 1	APOH
Q06033	41	25	342.4	3.63E-02	1.2	0.736	0.512–0.960	Inter-alpha-trypsin inhibitor heavy chain H3	ITI1H3
P00450	190	171	1431.8	4.90E-02	1.1	0.718	0.476–0.961	Ceruloplasmin	CP



therefore determined to be significantly different between the two groups. These proteins are given with the normalized abundance per sample in Supplementary Table S3. Of these proteins, 20 had higher levels in patients with long survival, and 11 proteins had higher levels in patients with short survival. The protein with the largest fold change (10.6) was CD5 antigen-like (CD5L), which displayed higher levels in patients with long survival. The proteins with the second and third largest fold changes (5.7 and 3.8, respectively) were keratin, type II cytoskeletal 2 epidermal (KRT2), and diphosphomevalonate decarboxylase (MVD). KRT2 had higher levels in patients with short survival, while MVD had higher levels in patients with long survival. We also found several proteins with roles in inflammation to have higher levels in patients with short survival. These included multiple complement components and C-reactive protein (CRP).

PCA and hierarchical clustering

PCA is used to determine the principle axes of abundance variation and is useful in identifying outliers. The PCA biplot is used to identify and visualize the relationship between two groups as it captures the differences between the groups. Figure 1 shows the PCA biplot when the samples were divided into groups based on patient survival and when all 140 proteins with two or more unique peptides were considered. The R2X of this PCA biplot was 0.4111.

The samples were also divided into three groups based on the years they were collected (2001...2004, 2006...2008, and 2010...2011) in order to investigate if protein expression could be affected by the time the samples were stored in the freezer. As seen in Supplementary Figure 1, the PCA biplot when all 140 proteins with two or more unique peptides were used shows no separation between

the three groups, confirming that storage time did not affect protein expression.

The heatmap generated when only the 31 proteins that passed the cutoff of a Mann-Whitney U test p-value of less than 0.05 between the two groups is given in Supplementary Figure 2. The figure shows that samples from patients with short or long survival form separate clusters, although several of the samples from patients with long survival clustered together with those from patients with short survival.

Pathway analysis

Pathway analysis by IPA found multiple canonical pathways that were enriched in this data set. The top five pathways enriched were the complement system, liver X receptor (LXR)/retinoid X receptor (RXR) activation, farnesoid X receptor (FXR)/RXR activation, acute-phase response signaling, and systemic lupus erythematosus signaling. The significantly different proteins that are part of these pathways are given in Supplementary Table 4 with their relevant information. The full list showing all canonical pathways enriched in this data set is given in Figure 2. Pathway analysis by IPA also generated networks of protein...protein interactions, and the top network is shown in Figure 3. This network was found to be associated with the following functions: developmental disorder, hereditary disorder, and immunological disease.

Discussion

In this study, we have used label-free proteomics to analyze the serum protein profiles of a cohort of PDAC patients with no apparent clinicopathological reasons for the differences seen in their survival (short or long). We discovered a total of 31 serum proteins whose levels

possible diagnostic and prognostic markers for PDAC. Levels of ceruloplasmin, a protein that transports serum copper, have previously been found to be significantly elevated in the serum of patients with various disseminated solid malignant tumors including lung, breast, head and neck, and gastrointestinal cancers.³⁴ Both ceruloplasmin and complement C3, as well as multiple other proteins detected in our study, including CRP and complement components, are acute-phase proteins whose concentrations change during inflammation.³⁵ As mentioned earlier, previous proteomic studies of PDAC have mainly focused on finding proteins of value for the diagnosis of PDAC. There is a paucity of studies that have profiled the serum or plasma proteome in order to discover candidates for new prognostic biomarkers.

Cancer has been linked to inflammation for many decades based on observations that tumors often arose at sites of chronic inflammation and the presence of inflammatory cells in biopsies. The inflammatory tumor microenvironment directly contributes to neoplastic progression, and tumor cells also co-opt signaling molecules of the innate immune system for their own use.^{36,37} PDAC has been recognized as an inflammation-driven cancer, with inflammation constituting a critical component of PDAC initiation and progression. The importance of inflammation is further supported by the fact that conditions such as chronic pancreatitis, which is characterized by chronic inflammation, significantly increases the risk of developing PDAC.^{38,39} The complement cascade contributes to both acute and chronic inflammation and has mostly been considered an effector of innate immunity. Since it is an important component of the inflammatory response and inflammation is involved in tumorigenesis and cancer progression, activation of the complement system in the tumor microenvironment contributes to pro-tumor processes. It has been shown to facilitate various aspects of cancer, including sustained proliferation, angiogenesis, invasion, and metastasis.^{40,41} A recent study by Law et al.⁴² used quantitative proteomics to analyze PDAC liver metastases and identified four distinct PDAC microenvironment subtypes, including an inflammatory subtype that did not respond to FOLFIRINOX treatment. This subtype was enriched for proteins related to complement activation and adaptive immune response, among others. The results from their study and our current study indicate that it would be of interest to identify a panel of serum and/or tumor tissue proteins that could be routinely used to classify inflammatory tumors, as well as other PDAC subtypes, as patients with these tumors may benefit from a more aggressive treatment regimen not including FOLFIRINOX. As we also identified multiple complement components whose levels were higher in PDAC patients with short survival (Table 1),

this further indicates that patients with inflammatory tumors have a very poor prognosis.

In our study, we also discovered that levels of CRP were higher in patients with short survival compared to those with long survival (Table 1). Elevated levels of CRP, an acute-phase protein, have previously been linked with poor prognosis in pancreatic cancer patients. In one study, the median survival of patients with an acute-phase response (CRP > 10 mg/L) was 66 days, while the median survival for patients without an acute-phase response was 222 days. The acute-phase response is also associated with metabolic disturbances, something that could account for the dysregulated levels of certain proteins involved in metabolism, such as several apolipoproteins, which were also seen in our study.^{43,44} We observed differences in serum levels of proteins such as CRP and other acute-phase proteins between PDAC patients with short and long survival, but levels of these proteins increase and decrease in general during inflammation. Elevated serum levels can therefore also be seen in the serum of patients with other diseases, meaning that these proteins are nonspecific for PDAC. While they could be used as prognostic markers for patients already diagnosed with PDAC, they most likely would not be helpful in the diagnosis of PDAC. However, we have previously shown that elevated levels of CRP predict worse survival in patients with resectable PDAC.⁴⁵ As CRP was found to have significantly higher levels in patients with short survival in our current study, this finding validates our results by indicating that we have accurately identified proteins whose levels differ between patients with short- and long-term survival.

Pathway analysis by IPA also revealed canonical pathways that were enriched in our data set. The top pathways were several involved in inflammation, such as the complement system and acute-phase response signaling (discussed above), as well as LXR/RXR and FXR/RXR activation, which are involved in metabolism. LXRs are nuclear receptors that function as cholesterol sensors, while FXRs are nuclear bile acid receptors. Both form heterodimers with RXRs and play important roles in regulating various aspects of metabolism. LXRs have also been established as modulators of not only lipid metabolism but also inflammation and immunity.^{46,47} Metabolic reprogramming and dysfunctional lipid metabolism are known to occur in cancer, which may explain the dysregulation of LXR/FXR and RXR/FXR activation seen in these PDAC patients.⁴⁸

One strength of this study is that the patients were carefully chosen and the only substantial difference between the patients in this study was in survival. In addition, the PCA biplot given in Supplementary Figure 1 confirms that the differences in protein expression seen between the short and long survival group are

actually due to the primary variable studied (survival time) and were not affected by factors such as time in storage. This study was limited by the fact that it did not include any samples from patients with inflammation due to other causes, such as pancreatitis, which could have helped remove inflammation-related proteins nonspecific for PDAC. If these had been removed, it may have been possible to discover proteins specific for PDAC that were present in low concentrations. We have, however, previously compared the proteome between patients with chronic pancreatitis and PDAC. Similar to here, levels of inflammation-related proteins were found to differ between the serum of these patients, and similar pathways were enriched in this data set as in our current study.²³ Our current study was limited by the small cohort of PDAC patients it utilized, due to the costly and tedious nature of sample preparation and analysis. While it identifies several possible candidates for prognostic markers, this is only the initial step, as candidate proteins would have to be validated in a larger cohort of patients before being of clinical use.⁴⁹ One way to do this is through the use of ELISAs, as ELISAs for many of the identified proteins are available. A validation project using ELISAs and aiming to analyze the top candidate proteins, including CD5L, MVD, CFHR5, and CRP as a control in a larger cohort of PDAC patients is currently underway.

In this pilot study, we identified 31 serum proteins whose levels differed significantly between samples from PDAC patients with short or long survival. These proteins displayed good AUROC values and could therefore be of value as prognostic markers for patients already diagnosed with PDAC. These types of prognostic markers could help to give more accurate prognostic information and subsequently aid in treatment decisions. By predicting the prognosis of PDAC patients, it would be possible to identify patients with good or poor prognosis. Patients tentatively predicted to have a good prognosis may be able to be treated with less aggressive chemotherapy regimens, which would spare them from undergoing unnecessarily harsh treatment, although the overall prognosis of PDAC is still poor. For the group of patients predicted to have a poor prognosis, more aggressive therapies may be needed if their general health allows it. Depending on the situation and outlook, patients predicted to have a poor prognosis may potentially benefit more from palliative care than radical surgery and aggressive treatment, as recovery time is often long after surgery. The findings of this pilot study therefore may help to improve the treatment and care of PDAC patients, although further validation of the proteins identified here is still needed.

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Author contributions

M.H., M.S., S.J., H.S., R.R., and C.H. conceived and designed the study. M.H., M.S., and S.J. acquired the data. M.H., M.S., and S.J. analyzed and interpreted the data. M.H. wrote the article. All authors read and approved the final version of the article.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

This study was approved by the Surgical Ethics Committee of Helsinki University Hospital (Dnro HUS 226/E6/06, extension TMK02 §66 17.4.2013) and was carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients included in this study.

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Supplemental material

Supplemental material for this article is available online.

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